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M. Ceperley; 09/788,286

Page 1

=> FIL MEDLINE

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L130	0	SEA	FILE=MEDLINE	ABB=ON	PLU=ON	L51 AND L129

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L51	616	SEA	FILE=MEDLINE	ABB=ON	PLU=ON	L46 AND L47
L127	42	SEA	FILE=MEDLINE	ABB=ON	PLU=ON	ETHANEDITHIOL OR ETHANE (2A) DITHIOL OR 540-63-6#
L128	861	SEA	FILE=MEDLINE	ABB=ON	PLU=ON	DITHIOL
L129	894	SEA	FILE=MEDLINE	ABB=ON	PLU=ON	L127 OR L128
L131	0	SEA	FILE=MEDLINE	ABB=ON	PLU=ON	L51 AND L129

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L128	861	SEA	FILE=MEDLINE	ABB=ON	PLU=ON	DITHIOL
L129	894	SEA	FILE=MEDLINE	ABB=ON	PLU=ON	L127 OR L128

L132 0 SEA FILE=MEDLINE ABB=ON PLU=ON L60 AND L129

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L65 13613 SEA FILE=EMBASE ABB=ON PLU=ON L63 OR L64
L66 61867 SEA FILE=EMBASE ABB=ON PLU=ON MASS SPECTROMETRY+NT,PFT/CT
L67 153 SEA FILE=EMBASE ABB=ON PLU=ON L65 AND L66
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L135 939 SEA FILE=EMBASE ABB=ON PLU=ON DITHIOL
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L63 9930 SEA FILE=EMBASE ABB=ON PLU=ON PHOSPHORYLATION+NT,PFT/CT
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L65 13613 SEA FILE=EMBASE ABB=ON PLU=ON L63 OR L64
L72 68784 SEA FILE=EMBASE ABB=ON PLU=ON PHOSPHOPROTEIN+NT,PFT/CT OR
PROTEIN PHOSPHORYLATION+NT,PFT/CT
L133 216 SEA FILE=EMBASE ABB=ON PLU=ON DITHIOL DERIVATIVE+PFT/CT
L134 63 SEA FILE=EMBASE ABB=ON PLU=ON ETHANEDITHIOL OR ETHANE (2A)
DITHIOL OR 540-63-6#
L135 939 SEA FILE=EMBASE ABB=ON PLU=ON DITHIOL
L136 979 SEA FILE=EMBASE ABB=ON PLU=ON L133 OR L134 OR L135
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L144 ANSWER 1 OF 2 EMBASE COPYRIGHT 2002 ELSEVIER SCI. B.V.
ACCESSION NUMBER: 1999130348 EMBASE
TITLE: Tyrosine phosphorylation/dephosphorylation controls capping ✓
of Fc.gamma. receptor II in U937 cells.
AUTHOR: Kwiatkowska K.; Sobota A.
CORPORATE SOURCE: Dr. A. Sobota, Nencki Inst. of Experimental Biology,
Department of Cell Biology, 3 Pasteur St., 02-093 Warsaw,
Poland. asobota@nencki.gov.pl
SOURCE: Cell Motility and the Cytoskeleton, (1999) 42/4 (298-314).
Refs: 64
ISSN: 0886-1544 CODEN: CMCYEO
COUNTRY: United States
DOCUMENT TYPE: Journal; Article
FILE SEGMENT: 029 Clinical Biochemistry
LANGUAGE: English
SUMMARY LANGUAGE: English

AB In the capping of cell-surface receptors two stages can be distinguished:
1) clustering of the receptors (patching) induced by cross- linking with
specific antibodies and 2) subsequent assembly of patches into a cap which
is driven by the actin-based cytoskeleton. We found that patching of
Fc.gamma. receptor II in U937 cells was correlated with tyrosine
phosphorylation of certain proteins, most prominently those of 130, 110,
75 and 28 kDa. The phosphotyrosine-bearing proteins were accumulated at
the receptor patches. Formation of the receptor caps was coincident with
dephosphorylation of these proteins. Inhibition of protein tyrosine
kinases with herbimycin A and genistein attenuated the protein tyrosine
hyperphosphorylation and blocked capping in a dose-dependent manner.
Phenylarsine oxide and pervanadate, inhibitors of protein tyrosine
phosphatases, also suppressed capping of Fc.gamma. receptor II in a
concentration- dependent fashion. Simultaneously, tyrosine
hyperphosphorylation of proteins occurred. In the presence of the tyrosine
kinase and phosphatase inhibitors the receptors were arrested at the
patching stage. In contrast, okadaic acid, a serine/threonine phosphatase
blocker, did not affect assembly of the receptor caps. The inhibitory
effect of phenylarsine oxide was rapidly reversed by dithiols,
2,3-dimercapto-1-propanoldithiol and dithiotreitol, and was coincident
with dephosphorylation of protein tyrosine residues. Extensive washing of
pervanadate-exposed cells also resulted in progressive restoration of the
cap assembly. Using streptolysin O-permeabilized cells we confirmed
regulatory function played by dephosphorylation of tyrosine residues in
capping of Fc.gamma. receptor II. Exogenous phosphatases, applied to
permeabilized cells in which activity of endogenous tyrosine phosphatases
was blocked, evoked dephosphorylation of protein tyrosine residues that
was accompanied by recovery of capping ability in the cells.

L144 ANSWER 2 OF 2 EMBASE COPYRIGHT 2002 ELSEVIER SCI. B.V.
ACCESSION NUMBER: 93007310 EMBASE
DOCUMENT NUMBER: 1993007310
TITLE: Identification of the adipocyte acid phosphatase as a
PAO-sensitive tyrosyl phosphatase.
AUTHOR: Shekels L.L.; Smith A.J.; Van Etten R.L.; Bernlohr D.A.
CORPORATE SOURCE: Department of Biochemistry, University of Minnesota, 1479
Gortner Avenue, St. Paul, MN 55108, United States
SOURCE: Protein Science, (1992) 1/6 (710-721).
ISSN: 0961-8368 CODEN: PRCIEI
COUNTRY: United States
DOCUMENT TYPE: Journal; Article

FILE SEGMENT: 029 Clinical Biochemistry
LANGUAGE: English
SUMMARY LANGUAGE: English

AB We have partially purified an 18-kDa cytoplasmic protein from 3T3-L1 cells, which dephosphorylates pNPP and the phosphorylated adipocyte lipid binding protein (ALBP), and have identified it by virtue of kinetic and immunological criteria as an acid phosphatase (EC 3.1.3.2). The cytoplasmic acid phosphatase was inactivated by phenylarsine oxide (PAO) ($K(\text{inact}) = 10 \text{ } \mu\text{M}$), and the inactivation could be reversed by the dithiol, 2,3- dimercaptopropanol ($K(\text{react}) = 23 \text{ } \mu\text{M}$), but not the monothiol, 2- mercaptoethanol. Cloning of the human adipocyte acid phosphatase revealed that two isoforms exist, termed HAAP.alpha. and HAAP.beta. (human adipocyte acid phosphatase), which are distinguished by a 34-amino acid isoform-specific domain. Sequence analysis shows HAAP.alpha. and HAAP.beta. share 74% and 90% identity with the bovine liver acid phosphatase, respectively, and 99% identity with both isoenzymes of the human red cell acid phosphatase but no sequence similarity to the protein tyrosine phosphatases (EC 3.1.3.48). HAAP.beta. has been cloned into Escherichia coli, expressed, and purified as a glutathione S-transferase fusion protein. Recombinant HAAP.beta. was shown to dephosphorylate pNPP and phosphoALBP and to be inactivated by PAO and inhibited by vanadate ($K(i) = 17 \text{ } \mu\text{M}$). These results describe the adipocyte acid phosphatase as a cytoplasmic enzyme containing conformationally vicinal cysteine residues with properties that suggest it may dephosphorylate tyrosyl phosphorylated cellular proteins.

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J. YAN ET AL
J. OF CHROMATOGRAPHY A
(29 MAY 1998) 808/1-2, 23-41.

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Review

Protein phosphorylation: technologies for the identification of phosphoamino acids

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Abstract

Protein phosphorylation plays a central role in many biological and biomedical phenomena. In this review, while a brief overview of the occurrence and function of protein phosphorylation is given, the primary focus is on studies related to the detection and analysis of phosphorylation both in vivo and in vitro. We focus on phosphorylation of serine, threonine and tyrosine, the most commonly phosphorylated amino acids in eukaryotes. Technologies such as radiolabelling, antibody recognition, chromatographic methods (HPLC, TLC), electrophoresis, Edman sequencing and mass spectrometry are reviewed. We consider the speed, simplicity and sensitivity of tools for detection and identification of protein phosphorylation, as well as quantitation and site characterisation. The limitations of currently available methods are summarised. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Reviews; Phosphoamino acids; Amino acids; Serine; Threonine; Tyrosine

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1. Introduction

Protein phosphorylation has increasingly become the focus of attention in cell biology and biomedical sciences because it is intimately involved in signal transduction, cell division and cancer [1–3]. Esterification of an amino acid side chain by the addition of phosphate, causes conformational changes in the protein and as a consequence altered protein activity or stability [4,5]. While it is now clear that protein phosphorylation acts as an on/off switch for many biochemical functions, only a subset of phosphoproteins is known, and the specificity of many of the enzymes involved in phosphate addition or removal remains obscure. The identification of phosphoproteins is possible by many direct and indirect means, but the localisation of the sites of phosphorylation remains a technical challenge. Here we address the status of knowledge of the characterisation of protein phosphorylation, particularly the identification of phosphoamino acids.

1.1. Nature and occurrence of phosphoamino acids

Protein phosphorylation is catalysed by a class of enzymes referred to as protein kinases, while removal of phosphate is controlled by a class of enzymes called phosphatases. The typical acceptors for eukaryotic phosphorylation are the hydroxyamino acids serine (Ser), threonine (Thr) and tyrosine (Tyr), while bacteria favor the use of histidine (His) and the carboxyamino acids [aspartic acid (Asp) and glutamic acid (Glu)] as phosphoacceptors [6]. However, many reports have shown that the abovementioned sites of phosphorylation are ubiquitous both in prokaryotes and eukaryotes [6]. Lysine (Lys) [7], arginine (Arg) [8] and cysteine (Cys) [9] have also

been identified as phosphate acceptors in both prokaryotes and eukaryotes.

Fig. 1 shows the structure of the phosphoamino acids known to be synthesised biologically. Although the order of chemical stability of free phospho-Ser [Ser(P)], phospho-Thr [Thr(P)] and phospho-Tyr [Tyr(P)] have been reported to differ [10–12], from a general point of view, the detection of *O*-phosphates after acid hydrolysis of proteins is possible because hydrolysis of the phosphomonoester bonds in the protein is considerably slower in acid than the hydrolysis of peptide bonds [13]. However, Tyr(P) contained in a protein, and as a single residue, is stable in alkali, and base hydrolysis is often recommended for the detection of phosphorylated Tyr(P) [14]. The properties of Tyr(P) differ significantly from Ser(P) and Thr(P) because of the hydrophobic benzene ring and the greater molecular mass. In addition, the β -elimination reaction which removes phosphate from Ser and Thr cannot occur on Tyr(P), so this analytical approach cannot be used.

Other phosphoamino acids, such as phospho-His [His(P)], phospho-Cys [Cys(P)] and phospho-Asp have been shown to be the phosphorylated reaction intermediates in several enzymes [15,16] and are unstable under the conditions of standard protein purification procedures [17]. It has been reported that His(P) is extremely acid labile [18]. Although there is an emerging awareness of critical roles of His(P) and Cys(P) in regulation, analytical techniques are limited, as detection is generally based on radiolabelling and an enzymatic approach, which is limited by the available enzymes (e.g., transmembrane histidine kinase [19]). We concentrate on the identification of Ser(P), Thr(P) and Tyr(P) in this review.

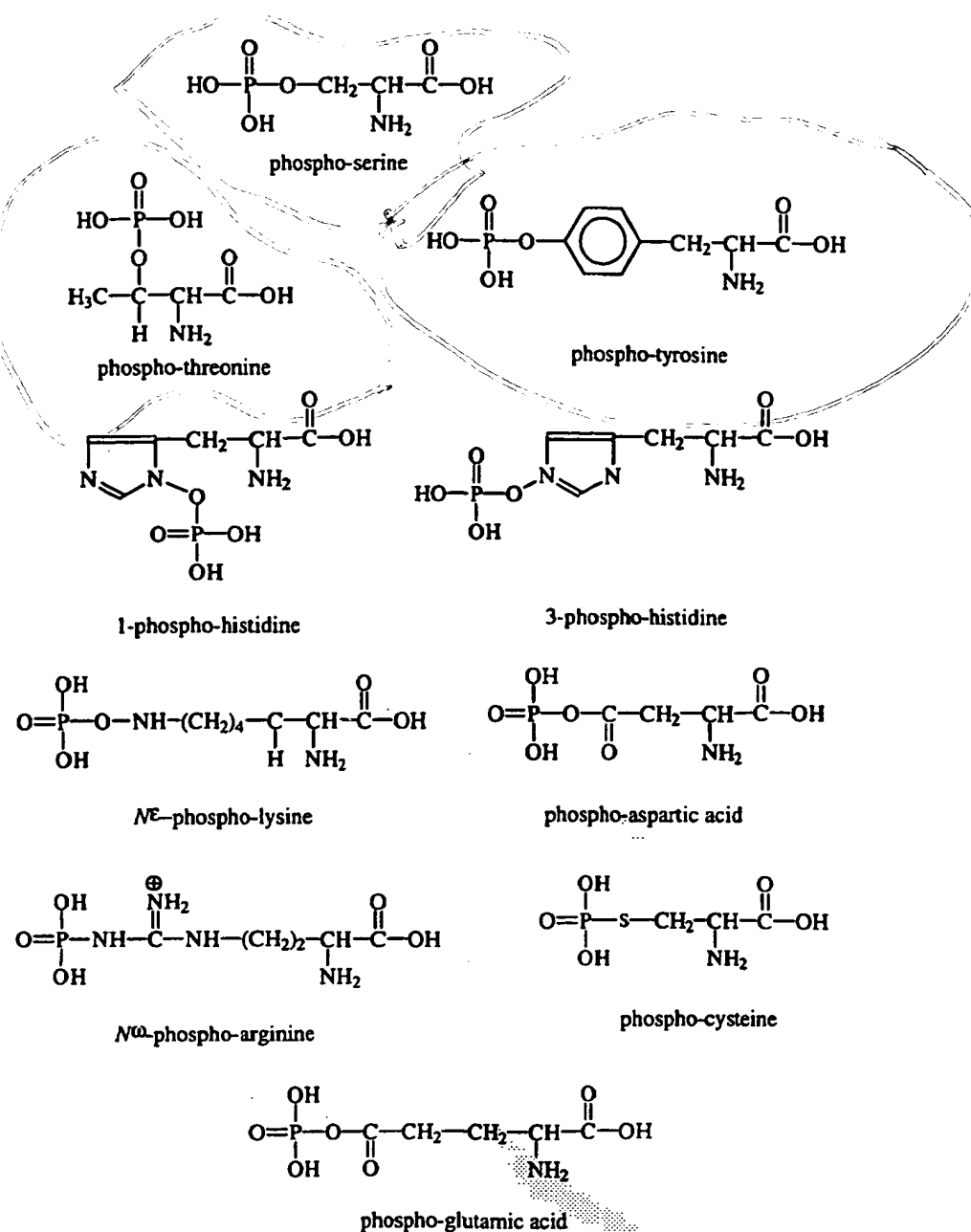


Fig. 1. The chemical structures of the phosphoamino acids known to be formed biologically.

1.2. Function of phosphorylation

Protein phosphorylation plays a central role in signalling pathways by providing the means for transducing extracellular signals and coordinating

intracellular events. It is a major regulator of the processes of gene expression and protein synthesis which determine cell growth, division or differentiation [6,20]. Since protein phosphorylation occurs by the reaction of protein kinases, a great body of

research has been focused on studies of the structure and function of protein kinases [21–23]. There is a World Wide Web site available on the Internet as a protein kinase resource (http://www.sdsc.edu/kinases/pk_home.html) which contains information on the protein kinase family of enzymes. Many of these

studies have focused on identifying the amino acid motif(s), the site(s) of phosphorylation in phosphoprotein(s) or synthetic peptides, which are recognised by specific kinases. Table 1 summarises the suggested motifs for Ser, Thr and Tyr phosphorylation by different protein kinases [24–51]. These

Table 1
Summary of phosphorylation sequence motifs recognised by various kinases

Sequence motif ^a	Enzyme	Protein (substrate)	Ref.
<i>Ser/Thr phosphorylation</i>			
Ser-Ser-Xaa-Ser(P)	Bone morphogenetic proteins receptor kinase	TGF-beta family mediator Smad1	[24]
Arg-Xaa-Arg-Yaa-Zaa-Ser(P)/Thr(P)-Hyd.	Protein kinase B	Synthetic peptide	[25]
Ser(P)-Xaa-His	Protein kinase C	Alpha 1 Na,K-ATPase	[26]
Ser(P)-Leu-Gln-Xaa-Ala	cGMP-dependent kinase	Lentivirus Vif proteins	[27]
Glu-Val-Glu-Ser(P)	c-Myb kinase	Venthrate c-Myb proteins	[28]
Arg-Xaa-Xaa-Ser(P)	Phosphotransferase	Serum response factor, c-Fos, Nur77 and the 40S ribosomal protein S6	[29,30]
Ser(P)/Thr(P)-Pro-Xaa (basic)	Cyclin-dependent kinase 2	Cyclin A or E	[31]
Pro-Xaa-Thr(P)-Pro-Xaa(basic)	Ceramide-activated protein kinase	Raf protein	[32]
Thr(P)-Leu-Pro	CAMP-dependent protein serine kinase	PII protein (glnB gene product)	[33]
Arg-Xaa-Ser(P)	Glycogen synthase kinase 3	cAMP responsive element binding protein	[34]
Ser-Xaa-Xaa-Xaa-Ser(P)	Autophosphorylation-dependent protein kinase	Myelin basic protein	[35]
Arg-Xaa-(Xaa)-Ser(P)/Thr(P)-Xaa-Ser/Thr	Ca ²⁺ /calmodulin-dependent protein kinase Ia	Peptide analogues	[36]
Hyd-Xaa-Arg-Xaa-Xaa-Ser(P)/Thr(P)-Xaa-Xaa-Xaa-Hyd.	Casein kinase	Bovine osteopontin, vitamin K-dependent matrix Gla protein from shark, lamb, rat, cow and human	[37,38]
Ser-Xaa-Xaa-Glu-Ser(P)	Casein kinase II	Bovine osteopontin	[39]
Xaa-Ser(P)/Thr(P)-Pro-Xaa	Proline-directed protein kinase	Tau protein	[40]
Ser-Pro-Arg-Lys-Ser(P)-Pro-Arg-Lys	Histone H1 kinase	Sea-urchin, sperm-specific histones H1 and H2B	[41,42]
Ser(P)-Pro-Lys/Arg-Lys/Arg-Lys-Ser(P)-Pro	Serine kinase	Murine neurofilament protein	[43]
<i>Tyr phosphorylation</i>			
Tyr(P)-Met-Asn-Met.	Phosphatidylinositol 3-kinase, in cytoplasmic tail	CD28 T cell costimulatory receptor	[44–46]
Tyr(P)-Xaa-Xaa-Met	Focal adhesion kinase, in cytoplasmic domain	Integrin beta-3	[47]
Tyr(P)-Met-Xaa-Met	Protein Tyr kinase, in cytoplasmic tail	Mast cell function-associated antigen	[48]
Asn-Pro-Xaa-Tyr(P),	Protein Tyr kinase	Synthetic peptides	[49]
Glu-Asp-Ala-Ile-Tyr(P)			
<i>Dual Thr and Tyr phosphorylation</i>			
Thr(P)-Xaa-Tyr(P)	Mitogen-activated protein kinase	p38 mitogen-activated protein	[50]
Thr(P)-Glu-Tyr(P)	Mitogen-activated protein kinase kinase	Mitogen-activated protein kinase	[51]

^a Xaa is any amino acid, Yaa and Zaa are small residues other than Gly, Hyd is hydrophobic amino acid residue Phe or Leu. The extended total environment of N- or C-terminal amino acids adjacent to the phosphorylation site is not stated.

phosphorylation sequence motifs have directly provided structural information on the binding site/domain and interaction between kinase and its substrate [25,47], and therefore provided an understanding of the kinase signal transduction pathway, and the regulation of phosphorylation. In addition, if a common phosphorylation sequence motif can be identified it will provide a useful indication of potential sites of phosphorylation for further study. In practice it is clear that there is no simple motif for protein phosphorylation. The suggested motifs (Table 1) for phosphorylation are certainly not highly conserved although charged amino acids are frequently found near the phosphorylation site. In addition, the motifs presented in the literature do not always cover both the N- and C-terminal amino acids adjacent to the phosphorylation site and thus the contribution of the total environment about the site cannot be determined. One possible strategy to identify phosphorylation motifs would involve the use of artificial neural networks to predict amino acid sequences that are phosphorylated. This approach has been used by the Centre for Biological Sequence Analysis at the Technical University of Denmark where servers are available to predict *O*-glycosylation in mammals and *Dictyostelium*, post-translational cleavage by picornaviral proteases and signal peptide cleavage sites (see <http://www.cbs.dtu.dk/services/>).

As well as basic housekeeping roles, protein phosphorylation is also used in many special functions. It is not the purpose of this review to be comprehensive about the function of phosphorylation, but the scope of its importance is demonstrated by the following examples. Ser phosphorylation has been shown to be important in enhancing transcriptional activation in vivo of the cellular p53 oncogene protein [52,53]. Thr phosphorylation influences catalytic activity in the catalytic subunit of cAMP-dependent protein kinase expressed in *Escherichia coli* [54]. In neural function, phosphoproteins appear to be particularly important in controlling neurotransmitter biosynthesis [55,56]. In the parotid gland, Tyr phosphorylation has a role in inducing the transition from stasis to active proliferation, and it has a potential role in mediating secretory function in the salivary glands [57]. In the cardiovascular system, Tyr phosphorylation of target proteins in the plasma membrane, the sarcoplasmic reticulum, and the

myofilaments of cardiac and vascular smooth muscle cells is known to be important in regulating contraction and relaxation [58]. Tyr phosphorylation is involved in the acute action of growth factors and vasoactive peptides on smooth muscle contractility [59] and in the regulation of lymphocyte function [60]. The literature abounds with many such important roles for protein phosphorylation.

2: Detection of protein phosphorylation

Detection of protein phosphorylation requires analysis at two levels: firstly, to determine if a protein is a phosphoprotein and secondly, to identify the amino acid(s) on which phosphorylation occurs.

A number of techniques are used in phosphorylation studies to detect phosphoproteins, such as the *in vivo* or *in vitro* incorporation of ^{32}P , the use of specific antibodies which recognise Ser(P), Thr(P) or Tyr(P) as part of the epitope, enzymic or chemical removal of phosphate, and analysis using chromatographic methods [high-performance liquid chromatography (HPLC), thin-layer chromatography (TLC)], electrophoresis, or mass spectrometry (MS). Often a combination of more than one of these techniques is necessary to achieve the best results.

All the techniques for the detection of phosphoamino acids described here are suitable for both *in vivo* and *in vitro* protein phosphorylation studies, i.e., whether phosphorylation is due to intracellular kinase activity (*in vivo*) or due to the reaction of a (semi) purified kinase(s) (*in vitro*).

In vivo phosphorylation studies identify authentic modification under physiological conditions, although in many cases the kinase(s) involved are unknown. With the advent of highly sensitive techniques, such as capillary electrophoresis (CE) and MS, even minor levels of *in vivo* phosphorylation can be detected. Often additional studies (e.g., with labelling) are needed to understand the dynamic status of phosphorylation *in vivo*.

2.1. Radiolabelling

The classical approach for the detection of phosphorylation is the incorporation of radiolabelled phosphate into phosphoproteins. The use of radiolabelled phosphate to measure the phosphorylation

state of enzymes began in 1956, with the demonstration that epinephrine and glucagon stimulated the phosphorylation of phosphorylase in dog liver slices [61]. This technique has been used in many systems and a selection of such studies [62–68] is given in Table 2. In vivo protein labelling involves the incubation of intact organisms, cells or tissues with [32 P]phosphate or [γ - 32 P]ATP in permeabilised cells. The next step involves the purification of the phosphorylated proteins without their further modification by cellular proteases, protein kinases, phosphatases, or protease/phosphatase inhibitors [69]. In vitro protein labelling involves incubation of a cell-free system containing a divalent cation (Mg^{2+} or Mn^{2+}), with a protein kinase [or cell fraction containing a protein kinase(s)] and [γ - 32 P]ATP as the substrate that serves as the phosphate donor for most protein phosphorylation reactions [69]. Usually the

starting point for analysis is the use of sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) to separate the proteins and exposure of dried gel to X-ray film to locate the 32 P-labelled phosphoproteins by autoradiography. Fig. 2 shows this scheme where radiolabelling is used as the first step to identify a candidate phosphoprotein. If the protein is already fully phosphorylated in vivo, further addition of phosphate will not occur and these phosphorylated sites will not be detected. Therefore, one can get a misleading impression of the in vivo phosphorylation status. Furthermore, protein kinases can have a broad specificity, and any correlation between phosphorylation observed in vitro should be further examined with studies in situ and/or in vivo to establish physiological relevance [70]. For example, it is possible that in a cell a kinase resides in a different cellular compartment to

Table 2
Some examples of phosphorylation studies using radiolabelling techniques in intact cells

Tissue	Agonist (antagonist) used	Regulation	Ref.
Rat hepatocytes	(Sp)-cAMPS and (Rp)-cAMPS, the stimulatory and inhibitory diastereomers of adenosine cyclic 3',5'-phosphorothioate	(Sp)-cAMPS increases the activity of pyruvate kinase and 6-phosphofructo-2-kinase/fructose -hepatocytes, and also blunted by (Rp)-cAMPS	[62]
Rabbit ileal epithelial cells	Ca^{2+} ionophore A23187 Theophylline Promethazine	Increasing the phosphorylation of 4–5 polypeptides Increasing the phosphorylation of three peptides, two of which had the same M_r and pI as the peptides altered by the Ca^{2+} ionophore Decreasing the phosphorylation of one peptide which increased by Ca^{2+} ionophore	[63]
Human monocytes	Phorbol 12-myristate 13-acetate	Stimulating the secretion of the lysosomal enzyme <i>N</i> -acetyl- β -D-glucosaminidase	[64]
Guinea pig parotid gland	Isoproterenol or carbachol	Increasing in the activities of lysophosphatidate acyltransferase, diacylglycerol kinase and diacylglycerol acyltransferase	[65]
Human platelets	Sp-5,6-dichloro-1- β -D-ribofuranosylbenzimidazole-3',5'-monophosphorothioate (Sp-5,6-DCI-cBiMPS) 8-(<i>p</i> -chlorophenylthio)-cAMP (8-pCPT-cAMP)	Both a potent and specific activator of cAMP dependent protein kinase A potent activator of cAMP-PK and cGMP-PK	[66]
Human platelets	8- <i>p</i> -chlorophenylthio-cGMP	Selectively activating cGMP-dependent protein kinase	[67]
Macrophages	Sodium fluoride + aluminum (AlF_4^-)	Activation of nitrogen activated protein kinase, phospholipase A2 and PtdIns-phospholipase C	[68]

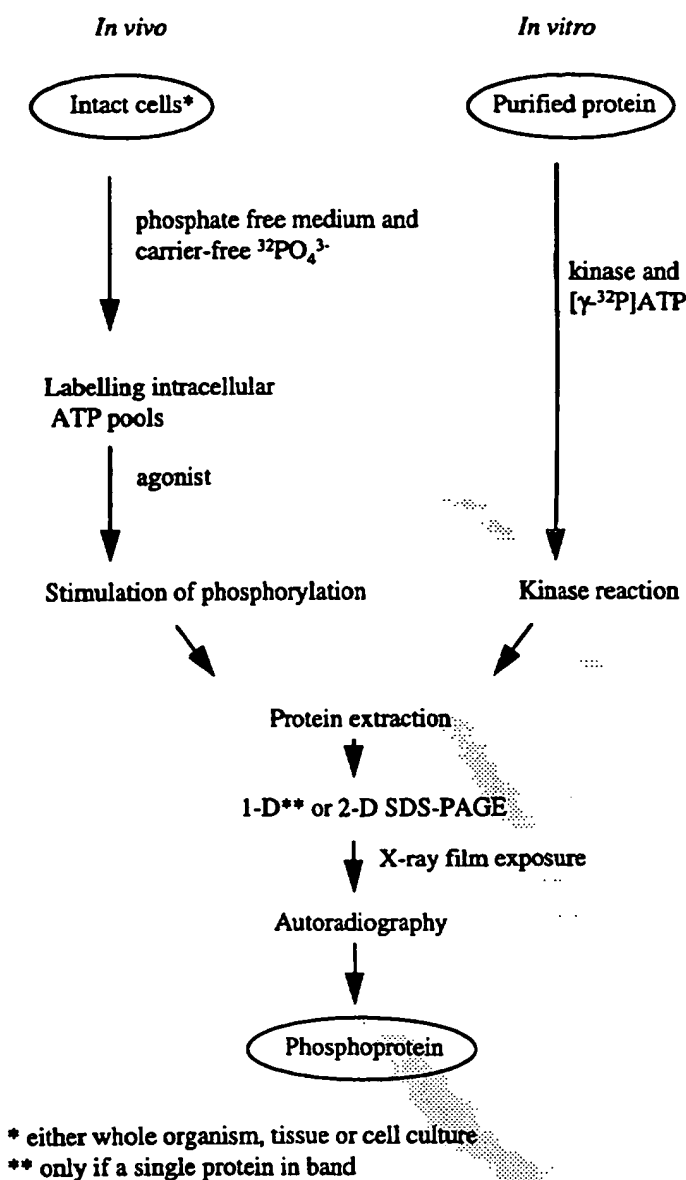


Fig. 2. Techniques for using radiolabelling to identify phosphoprotein(s).

the protein substrate, in which case the phosphorylation *in vitro* has little physiological significance.

Radiolabelling methods are very sensitive, and provide excellent resolution, although the *in vivo* method is limited to organisms or cells where label can be incorporated during growth. The major disadvantages are: (1) in order to obtain high specific

labelling, relatively large amounts of ^{32}P *in vivo* or high specific activity of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ *in vitro* are required; (2) the difference in metabolic phosphorylation/dephosphorylation rates results in a different proportion of ^{32}P incorporation onto Ser, Thr and Tyr residues which depends also upon the labelling time [10]. Hence one must be cautious in drawing

conclusions about the ratios of phosphorylated Ser, Thr and Tyr and indeed other kinds of protein phosphorylation; (3) constitutively phosphorylated proteins with a slow phosphate turnover rate may not be detected in short *in vivo* labelling studies.

2.2. Antibodies

Immunoblotting of cellular proteins with antibodies which recognise phosphoamino acid epitopes provides an alternative procedure to radiolabelling for detecting specific phosphoamino acids in phosphoproteins. This technique can be employed to monitor phosphorylation of proteins as they occur in sectioned material of organs or even whole animals. To identify which proteins are phosphorylated, proteins extracted from tissues can be displayed using gel electrophoresis, particularly two-dimensional (2D) SDS-PAGE, followed by Western blotting with the antibodies to detect the phosphoproteins.

Antibody detection is a sensitive method (fmol level) which is highly suitable for the detection of specific residues in individual phosphoproteins. A number of Ser(P)-, Thr(P)- and Tyr(P)-specific antibodies have been developed using specific phosphoproteins [71–73]. Interest in Tyr phosphorylation has resulted in the development of many Tyr(P)-specific antibodies [74–76]. The Tyr(P) phosphorylation state of several neural proteins, phosphorylated both *in vitro* and *in vivo*, has been studied successfully using antibodies generated against either the phospho- or dephospho-form of specific proteins (or corresponding peptides) [77–79]. The potential specificity of such antibodies generated against specific phosphorylation epitopes makes site-specific phosphorylation analysis feasible, providing the relevant antibodies can be prepared. However, while the phosphoamino acid is part of the recognition epitope, it is often not the defining component for the antibody which may recognise the phosphoamino acid only in a specific phosphoprotein or peptide context.

Monoclonal anti-Ser(P), anti-Thr(P) and anti-Tyr(P) antibodies are commercially available (e.g., from Sigma) for the detection of both free phosphoamino acids and phosphoamino acid-containing proteins. These antibodies were produced using Ser(P) or Thr(P) conjugated to keyhole limpet

hemocyanin (KLH) [80], and Tyr(P) to bovine serum albumin (BSA) [81]. No cross-reactivity was observed with the corresponding non-phosphorylated amino acid, or between phosphoamino acids. However, certain proteins known to contain phosphorylated amino acids were not recognised by these antibody due to steric hindrance of the recognition site [80,81]. Thus, the specificity and selectivity of antibody detection needs to be studied for individual proteins. Nevertheless, antibody detection provides a way of identifying specific phosphoamino acids at high sensitivity with a simple procedure.

2.3. Fluorescence labelling

Another general approach to the detection of protein bound phosphate groups is to derivatise them with a molecular label such as a fluorophore [82–84]. However, the idea of this being a general technique has not yet been fully explored since many of the chemistries used also label the amino acid side chain (Asp and Glu) and C-terminal carboxylic acid. Phosphate-specific fluorescence labelling with a reagent called BO-IMI, in which a BODIPY dye is attached to a reactive imidazole group, has been developed by Wang and Giese [85] for the detection of pepsin phosphorylation (a single site on Ser68) with sensitivity at the 10 μ g (3.6 nmol) level [86]. The hydrolytic instability of acylimidazoles (the side-product from labelling the carboxylic acid group with this reagent) accounts for the specificity of BO-IMI for labelling a phosphate as opposed to a carboxyl group [85], which was proven by the lack of labelling of albumin which is not phosphorylated. The yield for the specific labelling of Ser(P) is around 50%. BO-IMI also labels some other phosphate compounds, such as 5'-dADP, 5'-dATP, 5'-dGDP, glucose-6-phosphate, Thr(P), Tyr(P) and any phosphate on oligosaccharides and this could be a problem for some applications. There has not been a systematic study on the stability of this labelling. With the advent of high-resolution 2D gel electrophoresis, (phospho)protein(s) can be well resolved from nucleotides and oligonucleotides. In this area the development of a fluorescent labelling method would be useful as a sensitive and rapid screen for phosphoproteins without the use of radiolabelling.

3. Identification of phosphorylation by determination of phosphoamino acids

The techniques discussed above are used for detecting protein phosphorylation but do not give information on the specific location and quantitation of the phosphate on the protein. An alternative means for phosphoprotein detection is to screen for the presence of individual phosphoamino acids.

A scheme for the chemical identification of phosphoamino acids is presented in Fig. 3. Proteins (labelled or unlabelled) are partially hydrolysed to release the phosphoamino acids. The mixture of these phosphoamino acids is then separated by various techniques including column chromatography, TLC, or paper/gel electrophoresis. The steps used in this process are discussed in the following sections.

3.1. Hydrolysis

Partial acid, enzymatic or alkaline hydrolysis of the amide bonds of a protein can release phosphoamino acids. Theoretically, complete enzymatic hydrolysis would be the best approach for the accurate determination of the relative abundance and identity of phosphoamino acids within a protein. However, enzymatic degradation of proteins rarely goes to completion and this is a particular problem for phosphoproteins probably because the peptide bonds adjacent to phosphoamino acids are poor protease substrates [10]. In addition, the protease itself can contaminate further analyses.

More frequently, the protein sample is treated by acid or base hydrolysis. Proteolytic digestion followed by acid hydrolysis or alkaline hydrolysis may give superior yields of phosphoamino acids [14]. However, conditions which result in complete hydrolysis of the amide bonds are not satisfactory since the phosphate esters are also labile to both acid and base. The compromise of partial hydrolysis results in incomplete recovery of the phosphoamino acids. The rates of acid hydrolysis of both peptide and phosphoester bonds are affected by the nature of adjacent bonds [87]. Any method using hydrolysis to release phosphoamino acids must optimise the conditions to maximise the yield of the competing reactions of hydrolysis of the peptide bond and the phosphoester

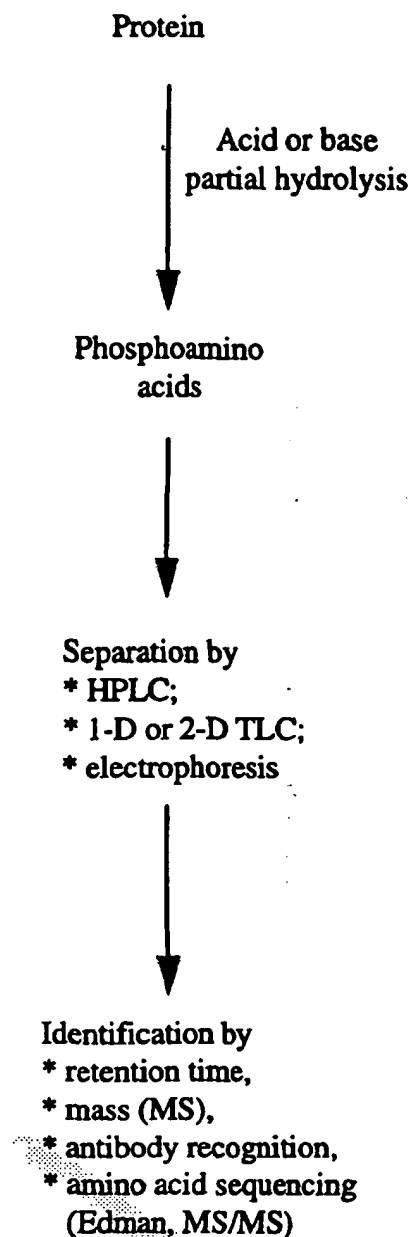


Fig. 3. Scheme for the chemical identification of phosphoamino acids.

bond. Surprisingly, there have not been any systematic studies on the comparative rates of cleavage of peptide bonds during acid or alkaline hydrolysis. This is an old technique for releasing phosphoamino acids, but it is becoming popular again due to the

adoption of methods of protein separation by SDS-PAGE and transfer to membranes [e.g., poly(vinylidene difluoride) (PVDF) blotting]. Partial hydrolysis of proteins separated by one-dimensional (1D) or 2D gel electrophoresis is a means of screening for phosphoproteins via identification of the presence phosphoamino acids [12]. The bonus is that the nature of the phosphoamino acid is determined (see Section 3.2).

3.2. Separation of phosphoamino acids

After partial hydrolysis or enzymic cleavage the released phosphoamino acids need to be separated for their analysis.

3.2.1. TLC and/or electrophoresis (one or two-dimensional)

For protein hydrolysates derived from an *in vitro* kinase reaction where $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (or GTP) is used as phosphoryl donor, Ser(P), Thr(P) and Tyr(P) can be separated and readily distinguished from the donor $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ by using 1D TLC or electrophoresis at pH 3.5 [88]. Thin-layer electrophoresis offers good resolution of these charged substances, such as phosphate, phosphoamino acids and phosphopeptides. These separated phosphorylated compounds can then be visualised and quantitated by radioactivity, and in this case it is not necessary to remove the non-phosphorylated amino acids and peptides.

When proteins are extracted from cells radio-labelled *in vivo* with $[\text{}^{32}\text{P}]\text{orthophosphate}$, the analysis of the protein hydrolysates can be complicated by the presence of radioactive contaminating phosphorylated compounds. For example, nucleoside monophosphates arising from RNA degradation and ribose phosphates generated by depurination of nucleotides during acid hydrolysis will also be radioactively labelled. Thus, 2D analytical systems are required to better separate the labelled products. These systems can be configured in various ways with chromatography and/or electrophoresis as the first and/or second dimensions (Fig. 4) [13]. With these methods nucleoside monophosphates can be separated from phosphoamino acids or phosphopeptides. However, comigration has been observed either between phosphopeptides, or between phos-

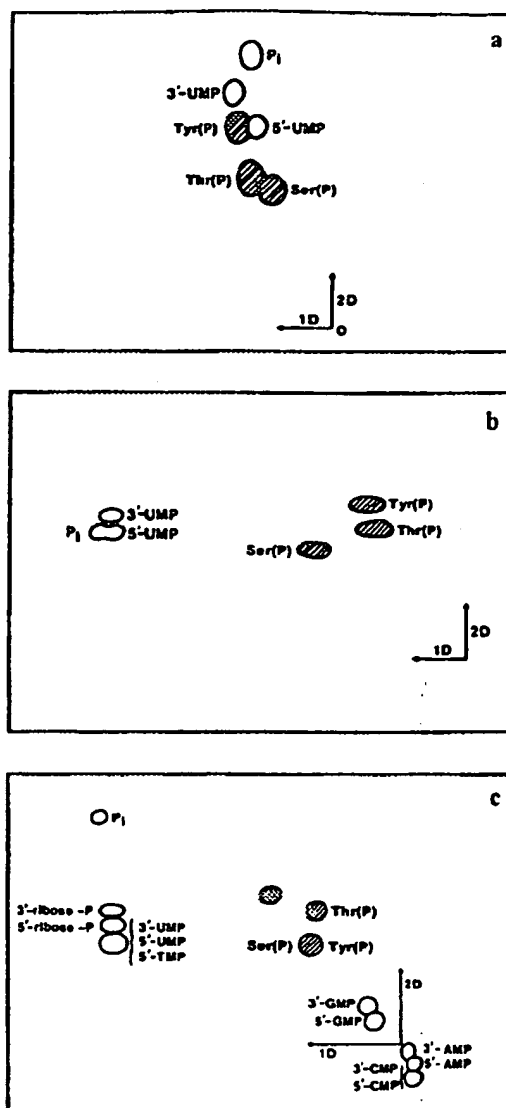


Fig. 4. Separation of phosphoamino acids derived from chemical hydrolysis by (a) double chromatography system: chromatographed with solvent A in the first dimension and with solvent B in the second dimension; (b) electrophoresis and chromatography system: electrophoresed at pH 1.9 in the first dimension and chromatographed with solvent A in the second dimension; (c) double electrophoresis system: electrophoresed at pH 1.9 in the first dimension and pH 3.5 in the second dimension. Solvent A=isobutyric acid–0.5 M NH_4OH (5:3, v/v); solvent B=2-propanol–11.4 M HCl–water (7:1.5:1.5, v/v/v); buffer at pH 1.9=7.8% (v/v) acetic acid and 2.5% (v/v) formic acid; buffer at pH 3.5=5% (v/v) acetic acid and 0.5% (v/v) pyridine. P_i =phosphate; AMP=adenosine monophosphate; CMP=cytidine monophosphate; UMP=uridine monophosphate; TMP=thymidine monophosphate; GMP=guanosine monophosphate.

phopeptide and non-phosphorylated peptides derived from the phosphoprotein during hydrolysis, and/or from the autodigested proteolytic enzyme in 2D TLC [89]. For further characterisation and identification it is necessary to separate these comigrating spots.

3.2.2. HPLC separation

Phosphoamino acids released by hydrolysis can be derivatised with chromophoric or fluorogenic reagents and separated, detected and quantitated by reversed-phase HPLC. Phosphoamino acid analysis is possible using the same chemistry as that used for derivatising amino acids for analysis, with modifications to the chromatography elution solvents, such as pH and hydrophobicity, to achieve optimal separation.

Table 3 summarises of a number of phosphoamino acid analysis methods using HPLC [12,16,90–97]. Over the years the separation and sensitivity of detection has improved. It is no longer necessary to radiolabel the proteins because of the improved sensitivity of detection using fluorescent derivatisation chemistries. A recent report using

Fmoc chemistry and reversed-phase separation offers high sample throughput and high sensitivity (30 pmol protein separated by 2D PAGE and electroblotted onto PVDF) making this technique suitable for routine analysis and mass screening of phosphoproteins [12]. If the purpose of phosphoamino acid analysis is simply to determine whether Ser, Thr or Tyr is phosphorylated, then this technique represents a fast, simple and reproducible technique to achieve this goal.

3.2.3. Mass spectrometry

The basic principle of MS approaches to the detection of protein phosphorylation is a mass difference of 79.983 u (HPO_3) (or multiples) between the phosphorylated and non-phosphorylated forms of a protein, or on a change in mass of a peptide after digestion with alkaline phosphatase. The technique has the significant advantages of being rapid, direct and does not require radiolabelling or derivatisation. Various ionisation methods have been used for the analysis of phosphopeptides and phosphoproteins by MS. These include fast atom bombardment (FAB)

Table 3
Summary of separation of phosphoamino acids using HPLC techniques

Ref.	Hydrolysis	Derivatisation	HPLC system	Sensitivity
[90]	4 M HCl, 110°C, 3 h	<i>o</i> -Phthaldialdehyde (OPA)	Anion-exchange column (Partisil-10 SAX resin), run time 60 min	10 pmol for standard phosphoamino acids; 17 pmol for myelin protein
[91]	6 M HCl, 110°C, 1–4 h	OPA	Cation-exchange column, (Durrum DC6A resin, 8 cm×0.6 cm I.D.), run time 80 min	50 pmol for standard phosphoamino acids; 4 nmol for protein
[92]	6 M HCl, 110°C, 1–4 h	None. Using UV absorption from amino acid at 243 nm	Ion-pair reversed-phase column (Spherisorb 5-ODS2), run time 10 min	200 pmol for standard phosphoamino acids; 25 nmol for protein
[93]	3 M KOH, 105°C, 4 h	OPA	Polymer-base reversed-phase column (Hamilton PRP-1), run time 15 min, His(P) can be analysed	30 pmol for standard phosphoamino acids
[94]	6 M HCl, 105°C, 2 h	UV absorption at 210 nm	Anion-exchange column (Partisil 10 SAX), run time 20 min	145 nmol histone, ^{32}P labelled
[95]	6 M HCl, 110°C, 3 h	9-Fluorenylmethyl chloroformate (Fmoc), 254 nm excitation, 313 nm emission	Anion-exchange column (Partisil 10 SAX), run time 35 min with other amino acids	479 pmol for Ser(P); 16–33 pmol for Thr(P)
[16]	6 M HCl, 110°C, 1 h	None. ^{32}P Cerenkov counting for protein sample, UV detection at 206 nm for standard phosphoamino acids	Anion-exchange column, Vydac Silica and Vydac oligonucleotide, run time 60 min	286 nmol for Thr(P); 605 nmol for Ser(P); 38 nmol for Tyr(P)
[96]	6 M HCl, 110°C, 2 h	4'-Dimethylamino-azobenzene-4-sulfonyl chloride, UV absorption at 460 nm	C_{18} Phenomenex Ultracarb 20 reversed-phase column	120 pmol for standard phosphoamino acids; 87 nmol for protein
[97]	6 M HCl, 110°C, 4 h	Phenyl isothiocyanate, UV absorption at 254 nm	C_{18} reversed-phase column (Waters), 30 cm long, run time 39 min including separation of other amino acids	10 pmol for standard phosphoamino acids; 72 pmol for PVDF bound β -casein
[12]	6 M HCl, 110°C, 4 h	Fmoc, fluorescent detection 270 nm excitation, 316 nm emission	C_{18} reversed-phase column (Hepersil, 15 cm long), run time 13 min, whole system was automated	fmol level for standard phosphoamino acids; 30 pmol for 2D SDS-PAGE separated, and PVDF blotted protein

[98,99]; electrospray ionisation (ESI) [99–101]; plasma desorption [102]; matrix-assisted laser desorption ionisation (MALDI) [103,104]. It has been difficult to distinguish sulfated and phosphorylated amino acids (addition of masses of 80.066 and 79.983 u, respectively), although this may be possible with new high mass accuracy MALDI instruments which have mass accuracy at 10 ppm. Also collision-induced dissociation (CID) in electrospray tandem MS [105] can be used to produce distinguishing fragment ions.

The developments in ESI-MS-MS and MALDI time-of-flight (TOF) have improved the sensitivity of the mass determination of peptides at the fmol level [89,106–109], and the site of addition of mass of a phosphate to an amino acid can now be resolved by sequencing the peptide using CID-MS-MS (see Section 3.4.3).

3.3. Quantitation of phosphoamino acids

Quantitation of phosphoamino acids is poorly developed in comparison with the detection and identification of phosphorylation. There is always an issue when fractionating cells to ensure that recovery of the target material is quantitative. A major challenge is the phosphatase susceptibility and chemical instability of phosphoamino acids, which are fully or partially degraded by prolonged treatment with strong acids and/or bases, or other chemical treatments, as well as by enzymatic breakdown.

In antibody analysis, detection relies on the Western blotting technique, which is generally not quantitative. In MS analysis, intensity of peak height is dependent upon ionisation of the protein/peptide which can be quantitatively related to the amount of material, if appropriate internal standards are used (e.g., enzyme inhibitors [110], and lipids [111]). However, internal standards are usually available only for material that has been extensively characterised. Other techniques such as HPLC, radioactive or fluorescent labelling and derivatisation, can potentially be used to quantitate phosphorylation. In addition, the recovery of isolation procedures of phosphoproteins is very important factor [69].

3.3.1. ^{32}P labelling of phosphoamino acids

^{32}P -labelled phosphoamino acids can be located

on TLC plates or 2D SDS-PAGE by autoradiography, and be quantitated theoretically by Cerenkov radioactivity counting. Sophisticated computer techniques are needed to manage the considerable amount of data generated from the autoradiographs. As discussed in Section 2.1 the determination of the specific activity of the ^{32}P in the cellular pools is subject to considerable uncertainty (see also Ref. [69]). The procedure is labor intensive and takes several days to perform, but the relative amount of radiolabelled phosphate can be determined in different proteins by this method. As with any radiolabelling technique, absolute quantitation is difficult as the specific activity of the pools of the relevant radiolabelled precursors are rarely well understood.

3.3.2. HPLC

HPLC is a separation technique whereby the amount of the relevant phosphoamino acid can be quantitated by comparison of the sample peak area with phosphoamino acid standards. However, the phosphoamino acids for HPLC analysis are invariably generated by partial protein hydrolysis during which phosphoamino acids are also partially degraded and/or are not fully released from the protein. However, we have found we usually recover 10–20% of the phosphoamino acid if there are more than 50 pmol, and this gives a guide to the level of phosphorylation and definition of the phosphoamino acids [12]. Although this technique [12] is semi-quantitative, it is particularly useful for prediction of the level on protein phosphorylation in a train of proteins separated by 2D PAGE to elucidate the cause of heterogeneity.

3.3.3. Chemical derivatisation and HPLC or Edman sequencing

An alternative way to quantitate Ser(P), Thr(P) and Tyr(P) is to chemically modify them to form a moiety which is stable to hydrolysis and to other harsh chemistries such as used in Edman protein sequencing and acid hydrolysis. Ser(P) can be modified by β -elimination of the phosphate in mild alkali, followed by the addition of ethanethiol, which yields the novel amino acid *S*-ethylcysteine [112]. Fig. 5 shows derivatisation of Ser(P) with ethanethiol. *S*-Ethylcysteine is stable during Edman degradation which can be assigned and quantitated

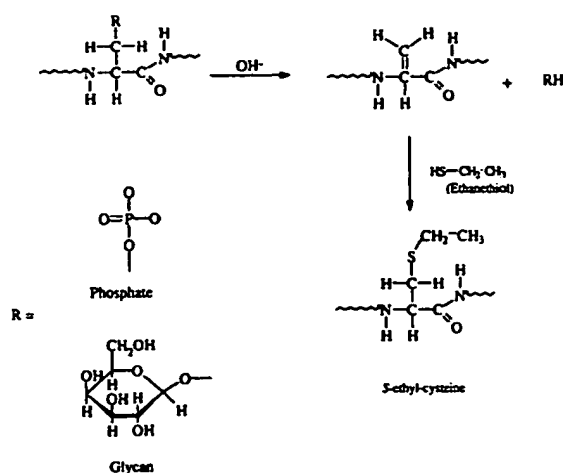


Fig. 5. The chemical reaction for β -elimination of *O*-linked phosphate or glycan from Ser, and the formation of the ethanethiol derivative, *S*-ethylcysteine.

during the sequencing [113,114]. This modification does not occur if Ser(P) is at the N- or C-terminus of the peptide, because the intermediates formed during the reaction are unstable [113].

Similarly, Thr(P) is modified chemically to β -methyl-*S*-ethylcysteine by β -elimination [115]. However, the phenylthiohydantoin (PTH) derivative liberated during Edman degradation has a limited solubility in the organic transfer solvents such as ethylacetate and is not soluble at all in butyl chloride, which is used in the older gas phase sequencers. A more polar extraction solvent, like trifluoroacetic acid (TFA), solves this problem when used in solid-phase sequence analysis [115].

Edman degradation is thus an effective method for quantitating Ser(P) and Thr(P) following β -elimination and derivatisation. These modified products also survive acid hydrolysis, and can be quantitated by reversed-phase HPLC analysis [116,117]. Using a similar approach, characterisation by capillary zone electrophoresis (CZE) and laser-induced fluorescence has also been used to quantitate the Ser(P) content of peptides and proteins at less than 75 amol [118]. However, it should be noted that other covalently modified Ser and Thr residues (e.g., by glycosylation), will also undergo β -elimination and derivatisation to form the same end product. Hence this method for Ser(P) and Thr(P) quantitation may overestimate their level in cases where glycosylation

is involved. In this case, it is necessary to prove that the modification is actually phosphorylation by another method, such as MS or ^{32}P labelling.

Differentiating between *O*-linked phosphorylation and glycosylation has become an important issue since the discovery of the *O*-GlcNAc modification to Ser/Thr [119–121]. Proteins can be phosphorylated or glycosylated at the same Ser or Thr residue. The ability to differentiate quantitatively between these two modifications will need to be developed if the significance of these alternate modifications to Ser and Thr is to be understood.

Tyr(P) cannot undergo β -elimination because the β -proton on its benzene ring cannot be easily eliminated. The anilinothiazolinone-Tyr(P)(ATZ) product in Edman degradation is insoluble in gas phase extraction solvents. For sequence analysis it requires covalent attachment of the peptide to a membrane and solid-phase sequence analysis combined with CE [115] or reversed-phase HPLC [122].

It is to be noted that the approach mentioned above involves chemical derivatisation followed by Edman sequencing, HPLC or CE quantitation techniques. In Edman degradation, during the acid or base delivery, the phosphate is β -eliminated and the resulting dehydro-amino acids rapidly form a dithiothreitol (DTT) adducts [115]. Thus if there is a fully modified Ser(P), it will exclusively form the PTH-DTT adduct of dehydroalanine, and no PTH-Ser adduct. PTH-Thr forms DTT adducts which are a series of diastereomers and are not easily detected. In contrast, *O*-HexNAc on Ser/Thr is stable in Edman degradation [123]. Thus, we believe this approach can be used for differentiation of Ser phosphorylation and glycosylation.

3.4. Sites of phosphorylation

Having detected a phosphoprotein and determined the nature of the phosphoamino acid, the location of the specific sites of phosphorylation is important information if the role of phosphorylation is to be understood.

3.4.1. Preparation of phosphopeptides

A number of techniques have been developed and used to locate phosphorylation sites in phosphoproteins. Phosphopeptide mapping has a long history

and all approaches employ the strategy of degrading the phosphoprotein chemically or enzymatically into small peptides in the initial step, followed by analysis of the composition and sequence of each fragment [105,124,125].

The classical approach to identifying sites of protein phosphorylation has been pretreatment of cells with [γ - 32 P]ATP *in vivo*. Radiolabelled phosphoproteins are separated by 2D SDS-PAGE, or combinations of electrophoresis/chromatographic analysis with detection by autoradiography after exposure to X-ray film [69,126]. Alternatively, the phosphoproteins are isolated by immunoprecipitation [127,128]. The radiolabelled proteins are then enzymatically digested directly on the gel pieces or after transfer to PVDF membrane. Specific isolation of phosphopeptides has been accomplished using immobilised metal affinity chromatography (IMAC) [129]. Fe^{3+} , Lu^{3+} , Sc^{3+} and Th^{4+} ions immobilised on chelating gels have exhibited a strong affinity for phosphorylated proteins and peptides, and selectively retain them from a complex mixture. The radiolabelled peptide fragments can then be eluted and sequenced by gas-phase Edman degradation [130].

2D separation of proteolytic digests of a purified phosphoprotein using chromatography as the first dimension and electrophoresis as the second dimension on TLC plates is a technique that is well suited to analysis of radiolabelled protein samples [13]. This is a sensitive technique that requires only a few disintegrations per minute (dpm) of metabolically labelled product. The TLC material, cellulose, is inert and the peptides can be recovered for secondary analysis, such as determining amino acid composition and sequence, or determining the presence and position of phosphoamino acid residues.

However, peptide-mapping experiments are tedious, especially when radiolabelling is involved, and they are often complicated by errors derived from sample preparation, leading to inconclusive results. Large sample losses can result from the *in situ* enzymatic cleavage reaction and in the recovery of peptides from the gel or PVDF membrane.

3.4.2. Edman degradation sequencing

As discussed in Section 3.3.3, except for Ser(P) at the N- or C- terminus of a protein/peptide, Ser(P) can be identified (and hence sites identified and

quantitated) using conventional gas phase Edman sequencing. Tyr(P) and Thr(P) can be analysed using solid-phase Edman sequencing. Another modified sequencing technique which applies not only to the quantitation of Ser(P) but also Tyr(P) and Thr(P), involves the measurement of 32 P-phosphate or other phosphorylated degradation products at the position of the phosphorylated residue during Edman degradation [131,132]. This is typically achieved by measuring the label present in the ATZ-Xaa transfer, as the eliminated phosphate is transferred with the amino acid. The alternative approach is to excise pieces of the sample membrane and count the 32 P following the cleavage of the candidate Ser/Thr/Tyr. However, if the washing of the membrane is exclusively non-polar, a lot of counts may remain on the membrane following elimination, making the carryover of label high in subsequent cycles. However, the detection of 32 P radioactivity is the only criterion for localising the phosphorylated amino acid by this approach. For example, if an amino acid were modified with a phosphorylated sugar this would be assumed to be direct phosphorylation. Obviously this technique requires a phosphoprotein which has been labelled *in vivo* or *in vitro*.

3.4.3. Mass spectrometry

As introduced in Section 3.2.3, the mass spectrometric method is a direct, rapid and powerful technique for protein phosphorylation studies. The localisation of phosphorylation sites can be conveniently divided into either peptide mass-mapping approaches [104,133,134] or direct MS sequencing [135]. Both parent scans [108,136] and *in-source* fragmentation with on-line LC-MS techniques [137,138] are widely used to identify modified peptides in protein digests.

Mass mapping of peptides to identify sites of phosphorylation relies solely on the mass determination of intact peptides enzymatically or chemically derived from phosphoproteins which contain a single Ser, Thr or Tyr. Phosphorylated peptide fragments will show a mass shift of 79.983 u increments (for each phosphate group modification) from the predicted peptide masses. The presence of a phosphate group can be detected or confirmed by MALDI-MS using mass determination before and after enzymatic dephosphorylation with alkaline phosphatase.

[104,139]. If two or more potential phosphorylation sites are present in a single peptide, the modified residue(s) can be identified by MS only if a secondary digest can hydrolyse the peptide bond between the possible sites. MALDI-TOF mass mapping has also been used successfully to identify phosphorylation sites without ambiguity in pmol quantities of phosphoproteins derived from biological sources [104,133,139]. Using an on-line enzyme reactor and ESI-MS, low pmol amounts of a Tyr(P) containing peptide were detected in a complex peptide mixture generated by proteolysis of a protein [140].

Most studies using MS–MS sequencing to locate the phosphorylated amino acid are reported on peptides with Tyr phosphorylation [89,141,142] because Tyr(P) is stable under the MS conditions, whereas Ser(P) and Thr(P) are less stable [89]. However, phosphorylation on Ser/Thr has been established using MS–MS [109,143–145].

Mass spectrometric sequencing approaches rely on fragmentation of peptide bonds during the analysis. From the fragment masses, the relative order of individual amino acids can be determined in the polypeptide chain. This method has the advantage of not requiring the protein sequence to be known in advance. The sensitivity has been extended into the low fmol range by analysing the peptides from protease digests by ESI-MS at high pH, and through the use of an ultra-low flow “nanospray” device [108,146]. MS–MS has been used to obtain the sequences of several of the identified phosphopeptides at the low fmol level [135].

With its high sensitivity and wide availability, MS will play an important role in protein phosphorylation studies in the detection of phosphorylation, and in the site specification of phosphoamino acids. However, it is yet to be able to quantitate the degree of phosphorylation at a specific site.

3.5. Proteome phosphorylation strategy

A fundamental change in protein chemistry is now occurring with emergence of the field of “proteomics” which aims to identify and characterise all proteins expressed by an organism or tissue [147]. Proteomics is based on the suite of technologies required to separate large numbers of

proteins, identify them and study their modifications. Proteome strategies have been proposed for the mass screening of proteins from biological samples using a hierarchy of technologies with the high-throughput/least cost methods followed by low-throughput/higher cost methods [148–153]. MS has become a key technology in the emerging field given its potential for rapid and highly sensitive identification of proteins [154–158].

The capability to analyse protein isoforms generated by post-translational modifications is a powerful feature of the proteome approach, allowing their impact on protein structure and function to be investigated. In our proteome studies we are applying the following strategy for analysing protein phosphorylation:

Proteome mapping: proteins are separated on a “proteome map” using 2D PAGE technology, and then identified utilising the mass screening approach [147]. Analysis of post-translational modifications normally focuses on a sub-set of proteins likely to be of most significance in the system under study as of course the analysis is necessarily more time assuming than the initial protein identification.

Detection and identification: the selected “spots” from the proteome map are analysed to determine the presence of phosphoamino acids utilising hydrolysis followed by HPLC [12]. This technology has the advantage of allowing high throughput together with sensitivity at the level of an “amido-black spot” (>10 pmol). Prefractionation techniques utilising metal affinity enriched chromatography [129] and narrow range immobilised pH gradient strip [159,160] allow high loading to handle low abundance proteins in regions of interest. Trains of spots where difference in isoelectric point is observed are of interest for possible differential protein phosphorylation.

Quantitation: for Ser(P)/Thr(P), chemical derivatisation followed by HPLC/Edman sequencing [113,115], and for Tyr(P) direct Edman sequencing [115,122] represent the only available techniques for quantitation in the context of proteome studies at this time. The level of expression under different conditions offers potentially important insights but these come at a cost as quantitation analysis is towards to low-throughput/slower end of the technology hierarchy.

Site characterisation: MS–MS sequencing is a powerful approach [108,136–138]. In the case of multiple sites, Edman sequencing is often the only practicable approach. Again, the techniques of site characterisation are at the low-throughput end of the proteome technology hierarchy but offers high sensitivity.

4. Conclusions

We have overviewed the key steps involved in protein phosphorylation studies: (1) detection – “are there any phosphoamino acids present in the protein?” i.e., “is the protein phosphorylated?”, (2) identification – “what is the phosphoprotein?”, (3) quantitation – “how much is present?” and (4) site characterisation – “where are the sites of individual phosphoamino acids?” Each step has been addressed strategically in terms of how it should be approached and the current status of available technologies reviewed (see summary in Table 4).

In conclusion, detection of protein phosphorylation can be achieved by radiolabelling, antibody recognition and phosphoamino acid chromatography, all with fmol sensitivity. Advanced protein chemistries, including Edman sequencing and MS–MS

analysis can then complete the characterisation of the phosphoprotein(s) and/or phosphoamino acid(s). Thus, it is now possible to rapidly study protein phosphorylation in a much wider range of proteins than previously thought possible.

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Table 4
Summary of technologies used in protein phosphorylation analysis

Technology	Detection	Phosphoamino acid analysis	Quantitation	Site determination
³² P labelling	+	–	+	–
Antibodies	+	+	–	–
MS (on whole protein)	+	–	+ ^a	–
Enzymic digestion				
(a) 1D or 2D TLC	+	–	–	+ ^b
(b) Derivatisation/Edman sequencing	+	+	+ ^c	+
(c) MS–MS	+	+ ^d	+	+ ^d
Hydrolysis				
(a) HPLC	+	+	+ ^e	–
(b) TLC	+	+	–	–

^a If appropriate internal standards are used.

^b If a single phosphorylation site is contained in the peptide.

^c Cannot differentiate between phosphorylation and glycosylation, if derivatisation is carried out prior to Edman sequencing.

^d Mainly Tyr(P), Ser(P) and Thr(P) are unstable and therefore difficult to study.

^e Only a semi-quantitative.

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